10/562059

SPECIFICATION

DISIALOUNDECASACCHARIDE CHAIN ASPARAGINE/FATTY ACID AMIDE

AND MEDICAL DRUG CONTAINING THE SAME

5

TECHNICAL FIELD

The present invention relates to asparagine-linked
disialoundecaoligosaccharide-fatty acid amide, and a composition or
a drug containing the same.

10

BACKGROUND ART

Influenza is an infectious disease which is prevalent in the
world almost every year. Many people are infected with influenza
every year despite the existence of vaccine and suffer from fever,
15 headache, musculus arthralgia and the like. Further they are
inflicted with complications, i.e. influenza brain inflammation and
encephalopathy and may die of the complication in the worst case.
The influenza virus is so feared despite the existence of vaccine
because influenza virus tends to cause gene mutation, leading to
20 change of antigen form so that the antibody increased in the body
by vaccine fails to recognize it, and the effect of vaccine is
lowered. As described above, even if it is predictable what type
of influenza will prevail every year, a problem is raised in that a
proper vaccine can not be rapidly supplied since a new type of
25 virus will be generated all at once. In this situation, it is
necessary to develop a highly effective drug which can show high
effects on various kinds of influenza viruses. In recent years,
the infection mechanism of influenza viruses have been clarified in

terms of molecule levels and gene levels due to marked progress of scientific technology. It is now under way to prepare various kinds of influenza virus infection inhibitors.
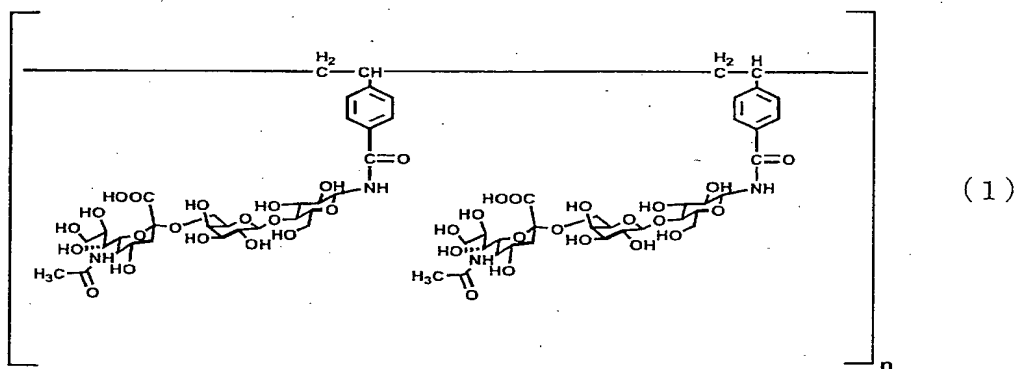
5   The infection of influenza viruses and the mechanism of growth are as follows. First, a virus is made to invade the living body. On approaching the host cell, a trimer of protein called hemagglutinin on the outer layer of the virus, becomes specifically bonded to a sialyl oligosaccharide, i.e. a receptor on the host cell. The sialyl oligosaccharide contains sialic acid at the end.

10   The sialic acid is chiefly involved in bonding to hemagglutinin. Then a membrane fusion occurs between the virus and the host cell so that the virus makes RNA infected with the host cell. Then, the virus gene is replicated therein for the advent of virus offspring. Then, a virus is germinated outside the host cell to complete the

15   propagation. In germination of a virus, hemagglutinin becomes bonded to sialyl oligosaccharide again. The sialic acid is separated from the sialyl oligosaccharide by sialidase, namely an enzyme, on the outer layer of the same virus, whereby germination can be achieved.

20   Recently new remedies have been developed for inhibiting the activity of sialidase reaction occurring at the last stage of virus infection. These two drugs are available under the names Zanamivir (trade name, Relenza, Glaxo Wellcome) and Oceltamivir phosphate (trade name, Tamiflu, Japanese Roche Corp.). However, these two

25   sialidase inhibitors are able to inhibit the infection at the last stage of infection and are less effective in inhibition because it acts after the growth of a virus has reached the peak. It is desirable to administer the drug within 48 hours after development
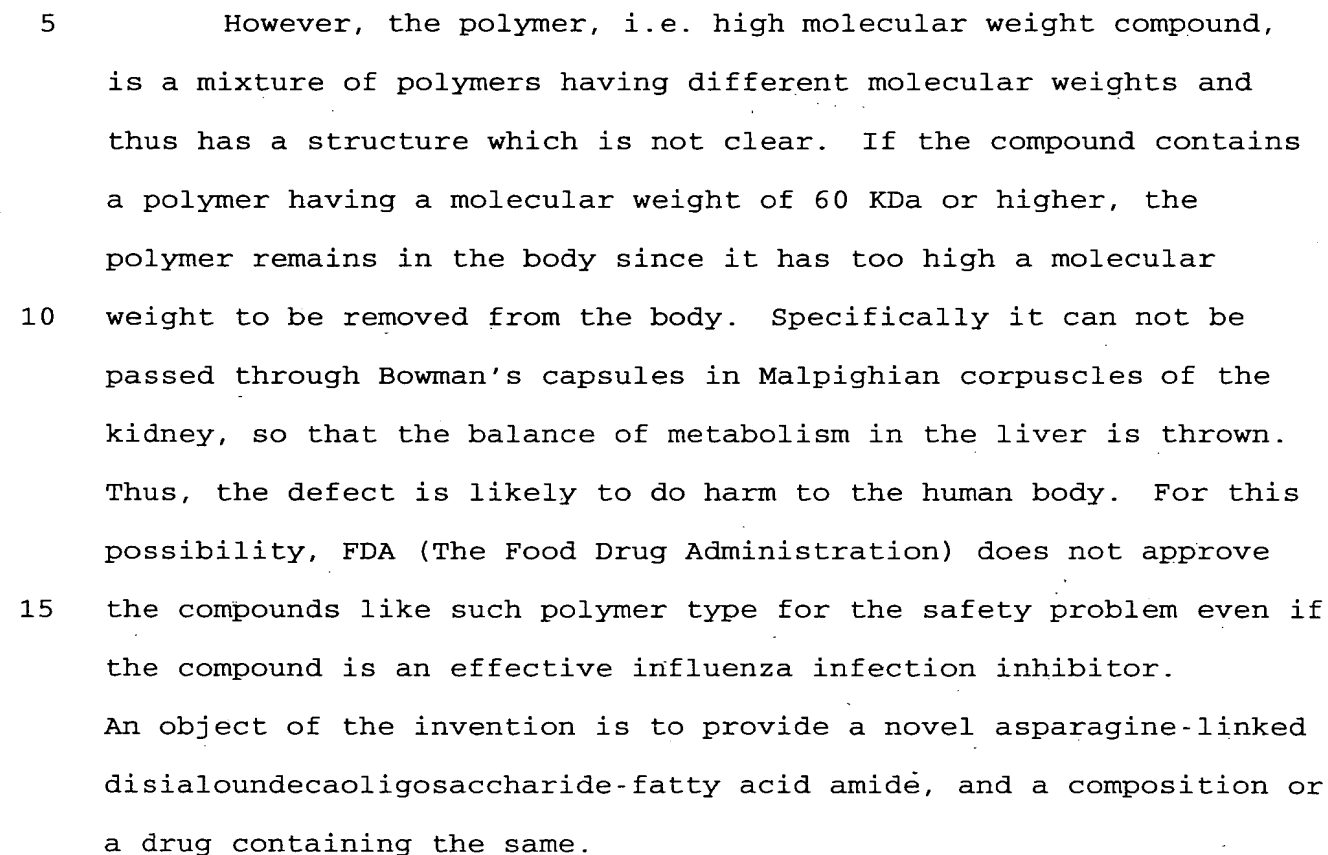
of infection. Therefore, a compound useful as an effective drug is demanded which inhibits the infection at the first stage at which the virus is bonded to the host cell.

It is already known that in the development of hemagglutinin
5   inhibitors, when a polyvalent sialyl oligosaccharide derivative having a number of sialyl oligosaccharides in a molecule shows a higher inhibiting activity against an influenza virus than a monovalent sialyl oligosaccharide derivative. This is because an affinity between a ligand and a receptor is more increased when
10   polyvalent molecules having a plurality of sialyl oligosaccharide derivatives are bonded thereto than when plural monovalent sialyl oligosaccharide derivatives are bonded to numerous hemagglutinins existing on the surface of the virus.

Up to date, a multiplicity of polyvalent sialyl
15   oligosaccharide derivatives have been developed with the expectation of such effect. Kanie et al prepared a styrene polymer (1) having sialyl lactose bonded thereto and researched the relation of the polymer with influenza. It was found that the polymer exhibits inhibition activity 1,000 times as high as fetuin
20   which is a sialyl oligosaccharide protein.

(1)

Whitesides et al synthesized a polyacrylamide polymer (2)

having a sialic acid derivative bonded and showed that the higher

the molecular weight of the polymer is, the higher the inhibition

activity is.

(2)

R=H or NeuAc derivative

5   However, the polymer, i.e. high molecular weight compound,

is a mixture of polymers having different molecular weights and

thus has a structure which is not clear. If the compound contains

a polymer having a molecular weight of 60 KDa or higher, the

polymer remains in the body since it has too high a molecular

10   weight to be removed from the body. Specifically it can not be

passed through Bowman's capsules in Malpighian corpuscles of the

kidney, so that the balance of metabolism in the liver is thrown.

Thus, the defect is likely to do harm to the human body. For this

possibility, FDA (The Food Drug Administration) does not approve

15   the compounds like such polymer type for the safety problem even if

the compound is an effective influenza infection inhibitor.

An object of the invention is to provide a novel asparagine-linked

disialoundecaoligosaccharide-fatty acid amide, and a composition or

a drug containing the same.

20   Another object of the invention is to provide a drug for

preventing and/or curing virus diseases such as influenza virus

infectious diseases, the drug having a high activity of inhibiting

the virus infection and/or the growth.
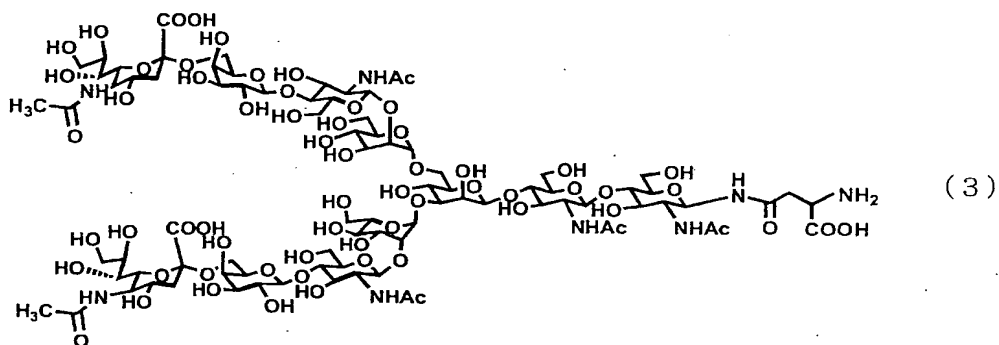
25   DISCLOSURE OF THE INVENTION

The invention provides an asparagine-linked disialoundecaoligosaccharide-fatty acid amide.

The invention provides a composition or a drug containing the asparagine-linked disialoundecaoligosaccharide-fatty acid amide.

5   The invention also provides a drug containing an asparagine-linked disialoundecaoligosaccharide.

The asparagine-linked disialoundecaoligosaccharide-fatty acid amide of the invention is a compound which can be prepared by reacting an asparagine-linked disialoundecaoligosaccharide with a

10   fatty acid.

The asparagine-linked disialoundecaoligosaccharide is an asparagine-linked oligosaccharide (3) shown below.

(3)

This asparagine-linked disialoundecaoligosaccharide can be

15   prepared, e.g., according to Reference Example 1 of Japanese Patent Application No.2002-196821.

Examples of the fatty acid constituting the fatty acid amide in the invention are aliphatic saturated or aliphatic unsaturated fatty acids having 6 to 32 carbon atoms and combinations of at

20   least two kinds thereof. Examples of the aliphatic saturated fatty acids include straight chain or branched chain hexanoic acid (caproic acid, 2-ethyl butanoic acid and so on), heptanoic acid (enanthic acid and so on), octanoic acid (caprylic acid, 2-

ethylhexanoic acid and so on), nonanoic acid (peralgonic acid and so on), decanoic acid (capric acid and so on), undecanoic acid, (undecylic acid and so on), dodecanoic acid (lauric acid, 2-ethyldecanoic acid and so on), tridecanoic acid (tridecylic acid

5   and so on), tetradecanoic acid (myristic acid and so on), hexadecanoic acid (palmitic acid and so on), octadecanoic acid (stearic acid and so on), eicosanic acid (arachic acid and so on), docosanoic acid (behenic acid and so on), and tetracosanoic acid (lignoceric acid and so on).

10   Examples of the aliphatic unsaturated fatty acids include straight chain or branched chain octenoic acid, decenoic acid, undecenoic acid, (undecylenic acid and so on), dodecenoic acid, octadecenoic acid (oleic acid, eraidic acid and so on), linoleic acid, linolenic acid and so on. Among these fatty acids, preferred

15   are fatty acids having 8 to 24 carbon atoms, and more preferred are those having 10 to 22 carbon atoms, especially preferred are decanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, octadecanoic acid, eicosanic acid, docosanoic acid and octadecenoic acid. Among them, most preferred are straight chain

20   fatty acids such as capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachic acid, behenic acid and oleic acid.

         The asparagine-linked disialoundecaoligosaccharide is reacted with the fatty acid preferably in the presence of a

25   reaction activator. Useful reaction activators are N-hydroxysuccinimide, N-hydroxybenzotriazole (HOBT) and so on.

         The asparagine-linked disialoundecaoligosaccharide is reacted with the fatty acid in a ratio of 1 mole of asparagine-

linked disialoundecaoligosaccharide and preferabyl about 0.1 to about 10 moles of the fatty acid. The reaction activator is preferably used in an amount of about 0.1 mole to about 10 moles per mole of asparagine-linked disialoundecaoligosaccharide. The

5   reaction is usually conducted at 0 to 80°C, preferably 10 to 60°C, and is usually completed in about 10 minutes to about 5 hours.

The asparagine-linked disialoundecaoligosaccharide-fatty acid amide thus obtained can be purified, e.g., by filtration, high performance liquid chromatography (HPLC) or the like. These

10   compounds can be identified by NMR, HPLC (ODS column), etc. in which the detection time in UV is determined.

The asparagine-linked disialoundecaoligosaccharide-fatty acid amide of the invention has both a hydrophilic group of asparagine-linked disialoundecaoligosaccharide and a hydrophobic

15   group of fatty acid having a long carbon chain, so that a micell can be easily created in an aqueous solution as shown in Fig.1, and a polyvalent asparagine-linked oligosaccharide derivative can be obtained which is of a low molecular weight type and which is clear in terms of molecular weight and structure.

20   In this way, a compound limited to a low molecular weight range can be produced more relatively easily than a polymer without difference in molecular weight by utilizing the production of a micell of monovalent molecules. Thus, for example, the obtained compound can exhibit a high inhibiting activity against an

25   influenza virus by the creation of a micell and forming polyvalent molecules.

The drug of the invention contains as an effective component the forgoing asparagine-linked disialoundecaoligosaccharide-fatty

acid amide. Further, the drug of the invention contains as an effective component the asparagine-linked disialoundecaoligosaccharide.

The drug of the invention is provided in the form of a pharmaceutical composition comprising the foregoing effective component and pharmaceutical additives (carriers, excipients and so on). Useful carriers include, for example, lactose, glycerin and so on. Useful excipients are, for example, lactose, glucose, sucrose, mannitol and so on. The amount of the effective component to be used in the drug is about 0.01 to about 95 wt%, preferably about 1 to about 80 wt%.

The administration route of the drug of the invention is not limited. An oral route or a parenteral route is employed. Parenteral administrations are, for example, intramascular, intravenous, subcutaneous and intraperitoneal administrations, applications of mucous membranes to nasal cavity, inhalation administration and so on. The form of the drug according to the invention is not limited. The form of the drug for oral administration is, for example, tablets, capsules, fine grains, powders, granules, solutions, syrups, etc. Examples of the formulations for parenteral administration are injections, drops, suppositories, inhalants, transmucosal absorbents, percutaneous absorbents, nose drops, ear drops, etc.

The form of the drug according to the invention, pharmaceutical additives to be used in the drug, the process for manufacturing the drug and the like are all within the skill of the art. The dosage of the drug according to the invention can be suitably selected in consideration of all of patients' sex, age,

weight, severity of disease, purpose of administration such as prevention or therapies, other complications, etc. The dosage is generally in the range from 0.001 μg per kg of weight per day to 1000 μg per kg of weight per day, preferably from 0.01 μg per kg

5 of weight per day to 100 μg per kg of weight per day.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows a micell of asparagine-linked disialoundecaoligosaccharide-fatty acid amide.

10 Fig.2 shows the chemical formula of each of asparagine-linked disialoundecaoligosaccharide-fatty acid amides prepared in Examples 1 to 4.

Fig.3 shows a reaction scheme for producing sialylated Dansyl-LacNAc.

15 Fig.4 shows the sialidase inhibition activity, based on the amount of sialic acid in each inhibitor.

Fig.5 shows the sialidase inhibition activity, based on the mole concentration of each inhibitor.

20 BEST MODE OF CARRYING OUT THE INVENTION

The invention will be described in greater detail with reference to Reference Example, Example and Test Example to which, however, the invention is not limited.

^{1}HNMR was determined by AVANCE 400(indicated as 400 MHz) of

25 Bruker. When a heavy solvent was used, the solvent peak was taken as the standard. The chemical shift was indicated as δ (ppm) and the binding constant was referred to as J(Hz). DC-Platten Kiesegel 60 F254 (Art 1,05715) manufactured by E. Merck was used for a

reaction detection (hereinafter referred to as "TLC"). Used as a
column for high performance liquid chromatography (HPLC) was
COSMOSIL PACKED ODS COLUMN (∅ 4.6 × 150 mm) manufactured by
Nacalai Tesque, Inc., Shodex C18-5B manufactured by Showa Denko K.K.

5   Used as a fluorescence spectrophotometer was FP 210
Spectrofluorometer manufactured by JASCO, Inc.
Reference Example 1
    Preparation of asparagine-linked disialoundecaoligosaccharide-1
        In 7.15 ml of a tris-hydrochloric acid-calcium chloride

10 buffer (TRIZUMA BASE 0.05 mol/l, calcium chloride 0.01 mol/l, pH
7.5) were dissolved a crude SGP (sialyl glycopeptide) (238.3 mg,
80.6 μmol), and sodium azide (4.77 mg, 80.8 μmol). Then Actinase-
E (53.8 mg) was added to the solution, and the mixture was allowed
to react at 37°C. After 165 hours, the completion of reaction was

15 confirmed by TLC and the reaction mixture was filtered. Then the
filtrate was freeze-dried. Thereafter the residue was purified by
gel filtration column chromatography (Sephadex G-25, ∅ 2.5 cm ×
100 cm) to give a desired asparagine-linked
disialoundecaoligosaccharide 1 (yield 100.5 mg, 53%).

20 ^{1}H-NMR(400MHz, D_2O)
    δ 5.22(1H, s, Man4-H1), 5.16(1H, d, J=9.6Hz, GlcNAc-H_1),
5.04(1H, s, Man4'-H_1), 4.86(1H, s, Man3-H_1), 4.70-4.68(3H, m,
GlcNAc2-H_1 GlcNAc5,5'-H_1), 4.53(2H, d, J=6.7Hz, Gal6,6'-H_1), 4.34(1H,
bd, Man3-H_2), 4.28(1H, bd, Man4'-H_2), 4.20(1H, bd, Man4-H_2), 3.03(2H,

25 dd, J=4.2Hz, 17.2Hz, Asn-βCH), 2.95(2H, dd, J=6.9Hz, 17.1Hz, Asn-
βCH), 2.76(2H, dd, J=4.6Hz, 12.4Hz, NeuAc7,7'-H_{3eq}), 2.14(18H, s×6,
-Ac), 1.80(2H, dd, J=12.2Hz, 12.1Hz, NeuAc7,7'-H3ax)
Example 1

Preparation of asparagine-linked disialoundecaoligosaccharide-
decanoic acid amide 3

Decanoic acid (22 mg, 127.6 μmol) was dissolved in 1 ml of
dimethylformamide. To the solution were added dicyclohexyl

5　carbodiimide (23.9 mg, 115.8 μmol) and N-hydroxysuccinimide (13.4
mg, 116.4 μmol). The mixture was reacted at room temperature.
After 6 hours, the reaction mixture was filtered, and the filtrate
was concentrated. Then the residue (decanoic acid succinimide) was
taken out. Then asparagine-linked disialoundecaoligosaccharide 1

10　(10.0 mg, 4.27 μmol) was dissolved in water (0.8 ml). Thereto
added was sodium hydrogencarbonate (1.4 mg, 16.7 μmol). Then
decanoic acid succinimide (3.2 mg, 11.8 μmol) dissolved in 1.2 ml
of acetone was added thereto. The mixture was stirred at room
temperature. After 180 minutes, it was confirmed by TLC that the

15　raw material disappeared. Then the solution was freeze-dried.
After it was freeze-dried, the residue was purified by gel
filtration column chromatography (Sephadex G-25, ∅ 1.0 cm ✕ 20 cm),
and freeze-dried. Thereafter it was purified by high performance
liquid chromatography (ODS column, ∅4.6 ✕ 150 mm, eluent: gradient

20　(60 minutes) water 100%→ acetonitrile 100%, flow rate 1.0 ml/min)
to give the contemplated decanoic acid-asparagine-linked
disialoundecaoligosaccharide 3 (yield 7.7 mg, 72%).
^{1}H-NMR(400MHz, D_2O)

δ 5.23(1H, s, Man4-H_1), 5.12(1H, d, J=9.6Hz, GlcNAc-H_1), 5.04(1H, s,

25　Man4'-H_1), 4.87(1H, s, Man3-H_1), 4.71-4.69(3H, m, GlcNAc2-H_1
GlcNAc5,5'-H_1), 4.59(1H, dd, J=4.5Hz, 8.0Hz, Asn-αCH), 4.53(2H, d,
J=7.9Hz, Gal6,6'-H_1), 4.34(1H, bd, Man3-H_2), 4.29(1H, bd, Man4'-H_2),
4.21(1H, bd, Man4-H_2), 2.88(2H, dd, J=4.4Hz, 15.5Hz, Asn-βCH),

2.78-2.70(3H, m, NeuAc7,7'-H$_{3eq}$, Asn-βCH), 2.34(2H, t, J=7.4Hz,

COCH$_2$), 2.14(18H, s×6, -Ac), 1.80(2H, dd, J=12.2Hz, 12.0Hz,

NeuAc7,7'-H$_{3ax}$), 1.71-1.60(2H, m, CH$_2$), 1.42-1.30(12H, m, CH$_2$),

0.95(3H, t, J=6.5Hz, CH$_3$)

5   Example 2

   Preparation of asparagine-linked disialoundeca-myristic acid

amide

      Myristic acid (22 mg, 96.3 μmol) was dissolved in 1 ml of

dimethylformamide. To the solution were added dicyclohexyl

10   carbodiimide (18.2 mg, 88.2 μmol) and N-hydroxybenzotriazole (11.9

mg, 96.7 μmol). The mixture was reacted at room temperature.

After 6 hours, the reaction mixture was filtered, and the filtrate

was concentrated. Then the residue (myristic acid benzotriazole)

was taken out. Then asparagine-linked disialoundecaoligosaccharide

15   1 (6 mg, 2.57 μmol) was dissolved in water (0.8 ml). Thereto added

was sodium hydrogencarbonate (0.86 mg, 10.3 μmol). Then myristic

acid benzotriazole (2.8 mg, 7.70 μmol) dissolved in 1.2 ml of

acetone was added thereto. The mixture was stirred at room

temperature. After 180 minutes, it was confirmed by TLC that the

20   raw material disappeared. Then the solution was freeze-dried.

After it was freeze-dried, the residue was purified by gel

filtration column chromatography (Sephadex G-25, ø 1.0 cm × 20 cm),

and freeze-dried. Thereafter it was purified by high performance

liquid chromatography (ODS column, ø4.6 × 150 mm, eluent: gradient

25   (60 minutes) water 100%→ acetonitrile 100%, flow rate 1.0 ml/min)

to give the contemplated myristic acid-asparagine-linked

disialoundecaoligosaccharide 4 (yield 5.3 mg, 81%).

^{1}H-NMR(400MHz, D$_2$O)

δ 5.23(1H, s, Man4-H$_1$), 5.12(1H, d, J=9.6Hz, GlcNAc-H$_1$), 5.04(1H, s, Man4'-H$_1$), 4.87(1H, s, Man3-H$_1$), 4.70-4.69(3H, m, GlcNAc2-H$_1$ GlcNAc5, 5'-H$_1$), 4.59(1H, dd, J=4.6Hz, 8.1Hz, Asn-αCH), 4.54(2H, d, J=7.8Hz, Gal6, 6'-H$_1$), 4.34(1H, bd, Man3-H$_2$), 4.29(1H, bd, Man4'-H$_2$), 4.20(1H, bd, Man4-H$_2$), 2.88(2H, dd, J=4.6Hz, 15.6Hz, Asn-βCH), 2.83-2.70(3H, m, NeuAc7,7'-H$_{3eq}$, Asn-βCH), 2.34(2H, t, J=7.4Hz, COCH$_2$), 2.14(18H, s$\times$6, -Ac), 1.80(2H, dd, J=12.1Hz, 12.1Hz, NeuAc7,7'-H$_{3ax}$), 1.71-1.60(2H, m, CH$_2$), 1.42-1.30(20H, m, CH$_2$), 0.95(3H, t, J=6.5Hz, CH$_3$)

Example 3

Preparation of asparagine-linked disialoundeca-stearic acid amide 5

Stearic acid (22.0 mg, 77.3 μmol) was dissolved in 1 ml of dimethylformamide. To the solution were added dicyclohexyl carbodiimide (14.4 mg, 69.8 μmol) and N-hydroxybenzotriazole (9.5 mg, 77.2 μmol). The mixture was reacted at room temperature. After 6 hours, the reaction mixture was filtered, and the filtrate was concentrated. Then the residue (stearic acid benzotriazole) was taken out. Then asparagine-linked disialoundecaoligosaccharide 1 (8.0 mg, 3.36 μmol) was dissolved in water (1.2 ml). Thereto added was sodium hydrogencarbonate (3.2 mg, 38.1 μmol). Then stearic acid benzotriazole (8.8 mg, 20.2 μmol) dissolved in 1.8 ml of acetone was added thereto. The mixture was stirred at 37°C. After 195 minutes, it was confirmed by TLC that the reaction has completed. Then the solution was freeze-dried. After it was freeze-dried, the residue was purified by gel filtration column chromatography (Sephadex G-25, Ø 1.0 cm $\times$ 20 cm), and freeze-dried. Thereafter it was purified by high performance liquid

chromatography (ODS column, Ø4.6 × 150 mm, eluent: gradient (60

minutes) water 100%→ acetonitrile 100%, flow rate 1.0 ml/min) to

give the contemplated stearic acid-asparagine-linked

disialoundecaoligosaccharide 5 (yield 6.2 mg, 69%).

5   ^{1}H-NMR(400MHz, D_2O)

δ 5.23(1H, s, Man4-H_1), 5.12(1H, d, J=9.5Hz, GlcNAc-H_1), 5.04(1H, s,

Man4'-H_1), 4.86(1H, s, Man3-H_1), 4.71-4.69(3H, m, GlcNAc2-H_1

GlcNAc5,5'-H_1), 4.59(1H, dd, J=4.6Hz, 8.2Hz, Asn-αCH), 4.54(2H, d,

J=7.8Hz, Gal6,6'-H_1), 4.34(1H, bd, Man3-H_2), 4.29(1H, bd, Man4'-H_2),

10   4.20(1H, bd, Man4-H_2), 2.88(2H, dd, J=4.7Hz, 15.5Hz, Asn-βCH),

2.78-2.70(3H, m, NeuAc7,7'-H_{3eq}, Asn-βCH), 2.34(2H, t, J=7.3Hz,

$COCH_2$), 2.16(18H, s×6, -Ac), 1.80(2H, dd, J=12.1Hz, 12.1Hz,

NeuAc7,7'-H_{3ax}), 1.71-1.60(2H, m, CH_2), 1.42-1.30(28H, m, CH_2),

0.96(3H, t, J=6.7Hz, CH_3)

15   Example 4

Preparation of asparagine-linked disialoundeca-behenic acid amide

6

Behenic acid (20.0 mg, 58.7 μmol) was dissolved in 3 ml of

dimethylformamide. To the solution were added dicyclohexyl

20   carbodiimide (10.3 mg, 50.0 μmol) and N-hydroxybenzotriazole (6.8

mg, 50.3 μmol). The mixture was reacted at room temperature.

After 19 hours, the reaction mixture was filtered, and the filtrate

was concentrated. Then the residue (behenic acid benzotriazole)

was taken out. Then asparagine-linked disialoundecaoligosaccharide

25   1 (5 mg, 2.1 μmol) was dissolved in water (1.2 ml). Thereto added

was sodium hydrogencarbonate (2.0 mg, 21.0 μmol). Then behenic

acid benzotriazole (8.8 mg, 18.9 μmol) dissolved in 1.8 ml of

acetone was added thereto. The mixture was stirred at 37°C. After

20 hours, it was confirmed by TLC that the reaction has completed. Then the solution was freeze-dried. After it was freeze-dried, the residue was purified by gel filtration column chromatography (Sephadex G-25, Ø 1.0 cm × 20 cm), and freeze-dried. Thereafter it

5 was purified by high performance liquid chromatography (ODS column, Ø4.6 × 150 mm, eluent: gradient (60 minutes) water 100%→ acetonitrile 100%, flow rate 1.0 ml/min) to give the contemplated behenic acid-asparagine-linked disialoundecaoligosaccharide 6 (yield 3.24 mg, 57%).

10 ^{1}H-NMR(400MHz, D$_2$O)

δ 5.23(1H, s, Man4-H$_1$), 5.12(1H, d, J=9.2Hz, GlcNAc-H$_1$), 5.04(1H, s, Man4'-H$_1$), 4.86(1H, s, Man3-H$_1$), 4.70-4.69(3H, m, GlcNAc2-H$_1$ GlcNAc5,5'-H$_1$), 4.59(1H, dd, J=4.5Hz, 8.0Hz, Asn-αCH), 4.54(2H, d, J=7.4Hz, Gal6,6'-H$_1$), 4.34(1H, bd, Man3-H$_2$), 4.29(1H, bd, Man4'-H$_2$),

15 4.21(1H, bd, Man4-H$_2$), 2.88(2H, dd, J=4.4Hz, 16.4Hz, Asn-βCH), 2.78-2.70(3H, m, NeuAc7,7'-H$_{3eq}$, Asn-βCH), 2.35(2H, t, J=7.3Hz, COCH$_2$), 2.13(18H, s×6, -Ac), 1.80(2H, dd, J=12.1Hz, 12.0Hz, NeuAc7,7'-H3ax), 1.71-1.60(2H, m, CH$_2$), 1.42-1.30(36H, m, CH$_2$), 0.95(3H, t, J=6.6Hz, CH$_3$)

20   Fig.2 shows chemical formula of each asparagine-linked disialoundeca-fatty acid amide of Examples 1 to 4.

Test Example 1

   Measurement of sialidase inhibition activity of inhibitor against influenza virus infection

25 (1) Sialylation of fluorescent-labelled N-acetyl lactosamine derivative

   It was investigated whether asparagine-linked disialoundecaoligosaccharide-fatty acid amide is really made into a

micell in an aqueous solution. For this purpose, the sialidase

inhibition activity was measured with respect to fluorescent-

labelled sialyl oligosaccharide to determine the IC_{50}. If the

asparagine-linked disialoundecaoligosaccharide 1, and four

5 synthesized glicolipid derivatives, respectively were made to

coexist with fluorescent-labelled sialyl saccharide 7 to determine

the sialidase inhibition activity and if the inhibition activity

is increased depending on the length of carbon chain, it is

presumably the effect of micell creation. First, fluorescent-

10 labelled sialyl oligosaccharide was produced. Dansyl group was

used for fluorescent-labelling. There was synthesized 6-[(N-

Dansyl)amino]-hexyl-O-β-D-galactopylanosyl-(1→ 4)-2-acetoamido-2-

deoxy-β-D-glucopyranoside (hereinafter referred to as "Dansyl-

LacNac") in which N-acetyllactosamine derivative was labelled with

15 Dansyl. Sialic acid was transferred to galactose using α (2,6)

sialyltransferase to give Dansyl-labelled sialyl oligosaccharide

(hereinafter referred to as "NeuAc-LacNAc-Dansyl").

Dissolved in cacodylic acid buffer (pH 6.0, 50 mM) were

Dansyl-LacNAc, CMP-sialic acid, α (2,6) sialyltransferase,

20 alkaline phosphatase and bovine serum alubmin. Then the solution

was reacted at 37˚C for 65 hours and purified by high performance

liquid chromatography, giving NeuAc-LacNAc-Dansyl 7 in a yield of

69%. ^{1}HNMR of the compound 7 was measured. It was confirmed that

3-position equatorial peak and axial proton peak (peaks specific to

25 sialic acid) existed in 2.74 ppm and 1.80 ppm. When it was

compared with ^{1}HNMR of raw material Dansyl-LacNAc, the 1-position

proton peak of galactose of compound 7 was shifted, as compared

with the peak of the raw material, to the higher magnetic field, so

that it was found that sialic acid was bonded to 6-position of galactose. In this way, the compound 7 was identified. The foregoing reaction is shown in Fig.3.

(2) Measurement of sialidase inhibition activity of asparagine-linked disialoundecaoligosaccharide-fatty acide amide

In an experiment wherein NeuAc-LacNAc-Dansyl is hydrolyzed with sialidase, the synthesized glycolipid derivatives 3, 4, 5 and 6, sialyl lactose, and asparagine-linked disialoundecaoligosaccharide 1 were added as the inhibitor, and each of the IC_{50} values was determined. The reaction conditions are as follows. Added to 100 μM of NeuAc-LacNAc-Dansyl was each of inhibitors in an amount of each of 10 μM, 100 μM and 300 μM (the solvent was a solution of HEPES buffer having bovine serum albumin dissolved therein). Then sialidase was added and the mixture was incubated at 37°C for 30 minutes. The decomposition ratio of fluorescent-labelled NeuAc-LacNAc-Dansyl was determined by high performance liquid chromatography and IC_{50} was calculated from the resulting values. The results were summarized with substrate by substrate. The concentration of the inhibitor required for inhibiting the enzyme reaction by 50% (IC_{50}) is plotted as ordinates. The graph of Fig.4 indicates the results of determining the IC_{50} with the amount of sialic acid in the reaction system. Since asparagine-linked disialoundecaoligosaccharide contains two sialic acids in a molecule, the measured values in determining IC_{50} are twice the amount of sialic acid. It is as shown in Fig.4.

The measured IC_{50} with respect to mole concentrations of these inhibitors were determined. The IC_{50} of inhibitors other than sialyl lactose is half the value of Fig.4 (Fig.5). It was

understood from the graph of Fig.4 that only when decanoic acid having the shortest carbon chain is bonded to asparagine-linked disialoundecaoligosaccharide 1 as a hydrophobic group, the value of IC_{50} is 3/4 the value of asparagine-linked

5   disialoundecaoligosaccharide 1 to which no hydrophobic group is bonded. This is presumably for the following reason. Carboxylic acid as a hydrophobic group is bonded to asparagine-linked disialoundecaoligosaccharide as a hydrophilic group, whereby the compound is made into a micell and numerous asparagine-linked

10   disialoundecaoligosaccharide are contained in one molecule. This means that the frequency of being caught by sialidase is increased, and the capability of inhibiting the removal of sialic acid by NeuAc-LacNAc-Dansyl is presumably enhanced. It was also found that the longer the carbon chain of hydrophobic group becomes

15   (decanoic acid → myristic acid → stearic acid→ behenic acid), the systematically lower the value of IC_{50} becomes. The reason may be that the longer the carbon chain of hydrophobic group becomes, the larger the surface area of the micell bodies become and the more the number of asparagine-linked disialoundecaoligosaccharide in the

20   surface of the molecule becomes, and the higher the capability of inhibiting the hydrolysis of sialic acid by NeuAc-LacNAc-Dansyl presumably is. It was also found from Fig.5 that only when the number of sialyl lactone is increased in the molecule only by one, the sialidase inhibition activity is enhanced, and the longer the

25   carbon chain is, the higher the effect is.

(3) Measurement of influenza virus inhibiting activity of asparagine-linked disialoundecaoligosaccharide-fatty acid amide

With addition of each of asparagine-linked

disialoundecaoligosaccharide-fatty acid amides of Examples 1 to 4 (derivatives 3, 4, 5 and 6 in Fig. 2) as an inhibitor, the influenza virus (hemagglutinin) inhibiting activity was determined. The reaction conditions are as follows.

5   The influenza virus used was A/New Caledonia/20/99 (H1N1).

The sample was inoculated with fertilized fowl ovum and the chorioallantois fluid was collected. After ultracentifuge purification and formalin inactivation, the obtained sample was used as as a purified virus antigen. Then after treatment with

10   ether, HA (hemagglutinin) fractions were collected to give HA split fractions.

1) Two mg of each of derivatives 3, 4, 5 and 6 of Fig.2 was dissolved in 1 ml of PBS.

2) The sample was used as it was or as diluted 2-fold, 4-

15   fold, or 8-fold with PBS.

3) 10 μl of each sample and 40 μl of virus diluted solution were placed and mixed in a 96-well U plate (Sanko Junyaku Co., Ltd.).

4) The mixture was allowed to stand at 4°C for 60 minutes.

20   5) 50 μl of PBS was added to the mixture to give 50 μl of 2-fold dilution solution.

6) The solution was adjusted to 0.5% of fowl hematid (Nippon Biotest Laboratories Inc.).

7) 50 μl of blood cell suspension was applied to each well

25   to give a mixture. The mixture was allowed to stand at 4°C for 60 minutes.

8) The measurement of influenza virus (hemagglutinin) inhibiting activity shows that the inhibiting activity was

exhibited when each derivative was used in the following amount:
190.0 μmol of derivative 3; 95.0 μmol of derivative 4; 47.5 μmol
of derivative 5; and 37.5 μmol of derivative 6.

5   INDUSTRIAL APPLICABILITY

The asparagine-linked disialoundecaoligosaccharide-fatty
acid amide and asparagine-linked disialoundecaoligosaccharide
according to the invention have a high virus infection inhibiting
activity and/or growth inhibiting activity and, for example,
10   exhibit an excellent effect as a drug for preventing and/or curing
virus diseases such as influenza virus infectious diseases.